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DECLASSIFICATION / DOWNGRADING SCHEDULE

ERFORMING ORGANIZATION REPORT NUMBER(S)

ITATION PAGE

	1b. RESTRICTIVE MARKINGS: N/A
	3. DISTRIBUTION / AVAILABILITY OF REPORT: Unlimited
	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 91 0119
NAME OF PERFORMING ORGANIZATION: Indiana University	6b. OFFICE SYMBOL (If applicable):
	7a. NAME OF MONITORING ORGANIZATION: AFOSR
ADDRESS (City, State, and ZIP Code): 4601 Central Avenue, Columbus IN 47203	7b. ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB DC 20332-6448
NAME OF FUNDING / SPONSORING ORGANIZATION: AFOSR	8b. OFFICE SYMBOL (If applicable): NL
	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR 90-0126
ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB DC 20332-6448	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102F	2312	A5	

TITLE (Include Security Classification)
The molecular anatomy of PFDA hepatotoxicity as studied by two-dimensional electrophoresis

PERSONAL AUTHOR(S)
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TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 12/15/89 TO 12/14/90	January 26, 1991	16

SUPPLEMENTARY NOTATION

COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
perfluoro-n-decanoic acid; two-dimensional electrophoresis; hepatotoxicity, rat liver

ABSTRACT (Continue on reverse if necessary and identify by block number)

Perfluoro-n-decanoic acid (PFDA) effects on protein expression in the rat liver were studied in rodents following *in vivo* exposure to PFDA levels above, below and at the LD-50. Two-dimensional whole-liver homogenate protein patterns were generated and compared to previous results. As before, numerous proteins were altered; some suppressed, some induced, but most were unaffected. In an effort to identify the altered proteins, further analysis of basic proteins by first-dimension NEPHGE revealed the induction of cytochrome P452 (lauric acid ω-oxidase) and enoyl-CoA hydratase. Induction of these and related enzymes confirms previously observed PFDA-induced peroxisome proliferation and lends strong support to the notion that PFDA blocks normal β-oxidation, causes fatty acid accumulation, and results in compensatory peroxisomal and mitochondrial ω- and β-oxidation Continued identification of other altered proteins will be undertaken to add to the metabolic paths affected by PFDA to further delineate its toxic mechanism.

DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified
NAME OF RESPONSIBLE INDIVIDUAL: Lt Col T J Carmany	22b. TELEPHONE (Include Area Code): 202-767-5021 / 22c. OFFICE SYMBOL: NL

Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

12 FEB 1991

Report AFOSR-90-0126

THE MOLECULAR ANATOMY OF PFDA HEPATOTOXICITY AS STUDIED BY TWO-DIMENSIONAL ELECTROPHORESIS

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3 Jan 1991

Annual Report for Period 15 Dec 89 through 14 Dec 90

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INTRODUCTION

Perfluoro-n-decanoic acid (PFDA) is a ten carbon straight-chain perfluorinated carboxylic acid whose surfactant properties give it and chemically similar compounds important commercial application. Previous studies of PFDA's toxicity showed that a single 50mg/kg i.p. dose (1) induces significant weight loss characterized by hypophagia (3), hepatomegaly, peroxisome proliferation (4-6), and delayed lethality in rats. Additional observations have led to the notion that PFDA interferes with normal β-oxidation of fatty acids resulting in an accumulation of fatty acids in the cell (2). As a result of this accumulation, peroxisomes proliferate as a prominent feature of compensatory β- (7,8) and ω-oxidative inductions (9,10).

Previous electrophoretic investigations conducted in this laboratory demonstrated that several proteins in the two-dimensional pattern from rat liver whole homogenates were variably affected by *in vivo* PFDA exposure (11,12). It was assumed that these protein alterations could be viewed as indicators of PFDA's toxic effect and in some way related to its hepatotoxic mechanism. With regard to these initial observations, the first-year's objectives of the present three year investigation were developed. Restated from the original proposal:

> *During the first year of the investigation we will incorporate the newly acquired ISO-DALT system and staining techniques into our recently renovated and expanded laboratory facilities, instruct the technical assistants in the use of this equipment, and confirm our earlier findings. Gels obtained from initial experiments will be used to train the Research Technician to use the Kepler Imaging System as per our collaborative arrangement with Lilly Research Laboratories, Eli Lilly and Company. The Research Technician as well as the undergraduate assistant will also learn how to make permanent records of the gel patterns using gel drying apparatus and newly acquired photographic equipment.*

The early two-dimensional electrophoretic studies of PFDA toxicity examined only those proteins with isoelectric points between pH 4-7. Generally, most proteins with pI's greater than 7 are poorly resolved under standard 2D conditions. Unfortunately, this basic group includes the cytochrome P-450 family as well as many of the β-oxidative enzymes. A secondary objective evolved as the initial phases of this investigation were underway:

> *Based on our most current understanding of PFDA's hepatic effects and because of early success in meeting the initial goals, the balance of our efforts were intended to analyze basic proteins associated with subcellular compartments as indicators of PFDA-mediated alterations in mitochondrial and peroxisomal lipid oxidation. Proteins from isolated microsomes and mitochondria were to be prepared and separated on two-dimensional gels using the large-scale ISO-DALT system. The hepatic proteins with basic pI's could thus be separated via nonequilibrium pH-gradient electrophoresis (NEPHGE) and later on second-dimension gel slabs in the presence of SDS.*

MATERIALS AND METHODS

Animal Care and Intoxication. Male Fisher-344 rats were obtained from Charles river Breeding Labs, individually housed, and maintained on rat chow and water *ad libitum*. Rats were injected with a single intraperitoneal dose of 2, 20, 50, or 100 mg PFDA/kg body weight; 75, 100, 150 mg PFOA/kg (eight carbon analog); or 3 successive daily doses of 400 mg clofibrate/kg. Matched control rats were vehicle injected and pair fed. After 8 days of exposure, rats were sacrificed by halothane anesthesia and livers removed.

Sample Preparation. Liver samples were removed from similar lobes in each rat; one minced and homogenized in 4 volumes of a lysis buffer containing 9M urea, 4% NP-40, 2% β-mercaptoethanol, and 2% ampholytes (Serva pH 9-11) pH 9.5 for ISO-DALT electrophoresis (13). Microsomal and mitochondrial fractions were prepared from a second sample homogenized in sucrose by differential centrifugation. These fractions were then solubilized in a lysis buffer containing 9M urea, 4% NP-40 detergent, 2% β-mercaptoethanol, 2% ampholyte (Serva pH 2-11) at pH 3.0 (13) for nonequilibrium pH-gradient electrophoresis (NEPHGE-DALT electrophoresis). After complete solubilization, samples were centrifuged at 110,000 x *g* and stored at -70°C.

Two-dimensional Electrophoresis. Using the Anderson ISO-DALT System (13), 30µL of solubilized protein was placed on each of 20 first dimension gels (25cm x 1.5mm) containing 4% acrylamide, 2% NP-40 (1% NP-40 and 1% dodecyl maltoside, NEPHGE), 2% ampholyte (ISO: BDH pH 4-8; NEPHGE: LKB pH 3.5-10) and electrophoresed (ISO: 27,000 VHr; NEPHGE: 4,000 VHr). Each first dimension gel was then placed on a second-dimension DALT slab gel (20cm x 25cm x 1.5mm) containing a linear 9-17% acrylamide gradient along with molecular weight standards on the gel margin. DALT gels were run for 18hr at 4°C and later stained with Coommassie brilliant blue G-250 (14). Protein patterns on some gels were electroblotted onto nitrocellulose for immunological identification of specific proteins. All stained gels and protein blots were photographed on a fluorescent light box with Kodak Panatomic-X film and printed on Ilford Multigrade III photographic paper.

RESULTS

Figure 1 (a-d) illustrates ISO-DALT gel photos illustrating the 2D protein patterns from rats treated with 20 and 50mg PFDA/kg body weight as well as the vehicle injected/pair-fed controls and *ad lib*-fed noninjected controls. By convention, the acidic (anodic) ends of the ISO gels were placed to the left and the basic (cathodic) ends to the right of the DALT gels. Thus the proteins with acidic pI's are located nearer the left side of the photos and basic proteins to the right. In the vertical dimension, high molecular weight (MW) proteins appear near the top while low molecular weight proteins migrate toward the bottom of the gels. Replicate gels containing carbamylated creatine kinase charge standard (15) and molecular weight standards (in kDa) are not shown although they were used to identify some of the proteins in this figure based on hepatic proteins previously characterized by others. Other protein spots in Figure 1 were given numerical identification based on their mobility relative to the MW standards and these reflect their apparent molecular weight. The protein indicated with arrows and numerical identification were altered consistently in five replicate samples while the encircled proteins were altered in some samples but not in others. Though neither densitometric nor image analyses

were conducted, it can be assumed that a change in spot volume or intensity corresponds to a quantitative change in protein expression, i.e. bigger spot, more protein and *vice versa.*

In a and b, no differences between pair-fed (b) and *ad lib*-fed treatments were detectable. In addition, two major cytoskeletal elements actin and tubulin (α and β) were unaffected by either PFDA dose (c and d). As the gel photos indicate, albumin (Al in a and d; MW 66kDa) spot intensity appears inversely related to PFDA dose. In contrast to albumin's reduction, the intensity of mitochondrial proteins M1, M2, and M3 (16) was increased by PFDA exposure.

Proteins with unknown identity were also altered by PFDA treatment. Foremost among those induced were those labelled 30.3, 40.8, 52.5, 69, and 80. Several proteins (29, 35, 36.3, and 38.7) were reduced in intensity. Of particular interest were alterations which appeared related to PFDA dose. These include dose-related reductions in protein 35, 36.3, and 38.7 and inductions in 30.3, 40.8, 52.5, and 69. Other altered proteins were up- or down-regulated without regard to PFDA dose. The results illustrated in Figure 1 represent the achievement of our initial objective; that is to reproduce earlier findings obtained using a small scale 2D approach with less sophisticated instrumentation.

The most recent results are illustrated in Figures 2-8. While Figure 1 illustrates only those proteins with isoelectric points between pH 4-7, approximately, Figures 2-8 illustrate the results of recent nonequilibrium pH gradient electrophoresis studies in which proteins with pI's greater than 7 were examined. This basic group of proteins includes many of the cytochrome P450 family and numerous enzymes of fatty acid oxidation. Figures 2-8 are labelled as to the cell fraction from which the proteins were prepared, their treatment, and dose. Proteins conclusively identified by immunological means or tentatively based on literature values (17) were labelled as follows: (A) peroxisomal/microsomal enoyl-CoA hydratase (bifunctional enzyme; 80kDa); (B) cytochrome P452 (lauric acid ω-oxidase; 52kDa); (C) peroxisomal enoyl-CoA hydratase polypeptide I (39.5kDa); (D) peroxisomal enoyl-CoA hydratase polypeptide II (35kDa); (E) 3-ketoacyl-CoA thiolase (44.5kDa); (F) mitochondrial enoyl-CoA hydratase (crotonase; 27kDa); and (G) unknown (32kDa). Only the identity of cyt P452 has been immunologically confirmed; others will soon be investigated.

DISCUSSION

These results demonstrate significant PFDA-induced effects on the 2D protein pattern of both rat liver homogenates and specific cell fractions. Previous investigations have concluded that PFDA hepatotoxicity involves compromised lipid metabolism, altered mitochondrial function and altered plasma membrane phenomena. All are thought to be related to PFDA's integration into cellular membrane systems. Recent notions suggest PFDA blocks normal β-oxidation of fatty acids resulting in fatty acid accumulation. The present results, especially the most recent NEPHGE-DALT studies of microsomal and mitochondrial/peroxisomal protein patterns support the idea that in order to compensate for the β-oxidative impairments, peroxisomal, microsomal, and mitochondrial β- and ω-oxidative enzymes are induced (Fig. 1: M1, M2, M3 and Figs. 2-8 A-F). In this respect PFDA parallels clofibrate, a known hypolipidemic agent and peroxisome proliferator.

These data serve to confirm previously held notions regarding PFDA's hepatotoxicity. It is clear that the 2D-PAGE approach is a significant tool in the elucidation of xenobiotic mechanisms. As a result of these first-year studies, the research plan is well grounded to

support the accomplishment of future objectives, the identification of many of the protein alterations involved in PFDA hepatotoxicity, elucidation of the underlying mechanism, the contribution of these data to a global liver protein database, and the future application of this approach to other tissues and other toxic chemicals of interest to the Air Force.

Publications resulting from this effort:

Hepatic protein alterations following perfluorodecanoic acid exposure in rats. Witzmann, F.A. and Parker, D.N. **Toxicology Letters** (in press) 1991.

Dodecyl maltoside detergent improves the resolution of hepatic membrane proteins on two-dimensional gels. Witzmann, F.A., Jarnot, B.J., and Parker, D.N. **Electrophoresis** (in preparation) 1991.

Effect of perfluoro-n-decanoic acid on hepatic lipid oxidative enzymes: A two-dimensional electrophoretic analysis. Witzmann, F.A., Jarnot, B.J. and D.N. Parker. **Toxicology Letters** (in preparation) 1991.

Papers presented at scientific meetings:

Two-dimensional electrophoretic analysis of PFDA hepatotoxicity. Witzmann, F.A., DelRaso, N. and George, M. at the 29th Annual Meeting of the Society of Toxicology, February 12-16, 1990 in Miami Beach FL. Published as an abstract in **The Toxicologist** 10:251, 1990.

Induction of cytochrome P452 in liver cell fractions by perfluoro-n-decanoic acid: an electrophoretic analysis. Witzmann, F.A. and D.N. Parker. Presented at the North American Meeting of the International Society for the Study of Xenobiotics, October 1990 in San Diego CA.

Two-dimensional protein electrophoresis: Hepatotoxic applications. Witzmann, F.A. Presented at the Society of Environmental Toxicology and Chemistry Annual Meeting, November 1990 in Alexandria VA.

Induction of an 80kDa protein in rat liver homogenates and cell fractions by perfluoro-n-decanoic acid. Witzmann, F.A. and Parker, D.N. Presented at the 30th Annual Meeting of the Society of Toxicology, February 25-March 1, 1991 in Dallas TX.

Hepatotoxicity of perfluorinated compounds in male rats: 2D-PAGE analysis. Witzmann, F.A. and Parker, D.N. To be presented at the International 2D-PAGE Conference, London, England July 16-19, 1991.

Perfluoro-n-decanoic acid toxicity in the rat liver: Two-dimensional electrophoretic analysis. Witzmann, F.A. To be presented as an invited selected topics speech as part of the section, Molecular Pathology: Applications to the Study of Xenobiotic Effects in Animal Testing at the 43rd National Meeting of the American Association for Clinical Chemistry, July 28-August 1, 1991 in Washington DC.

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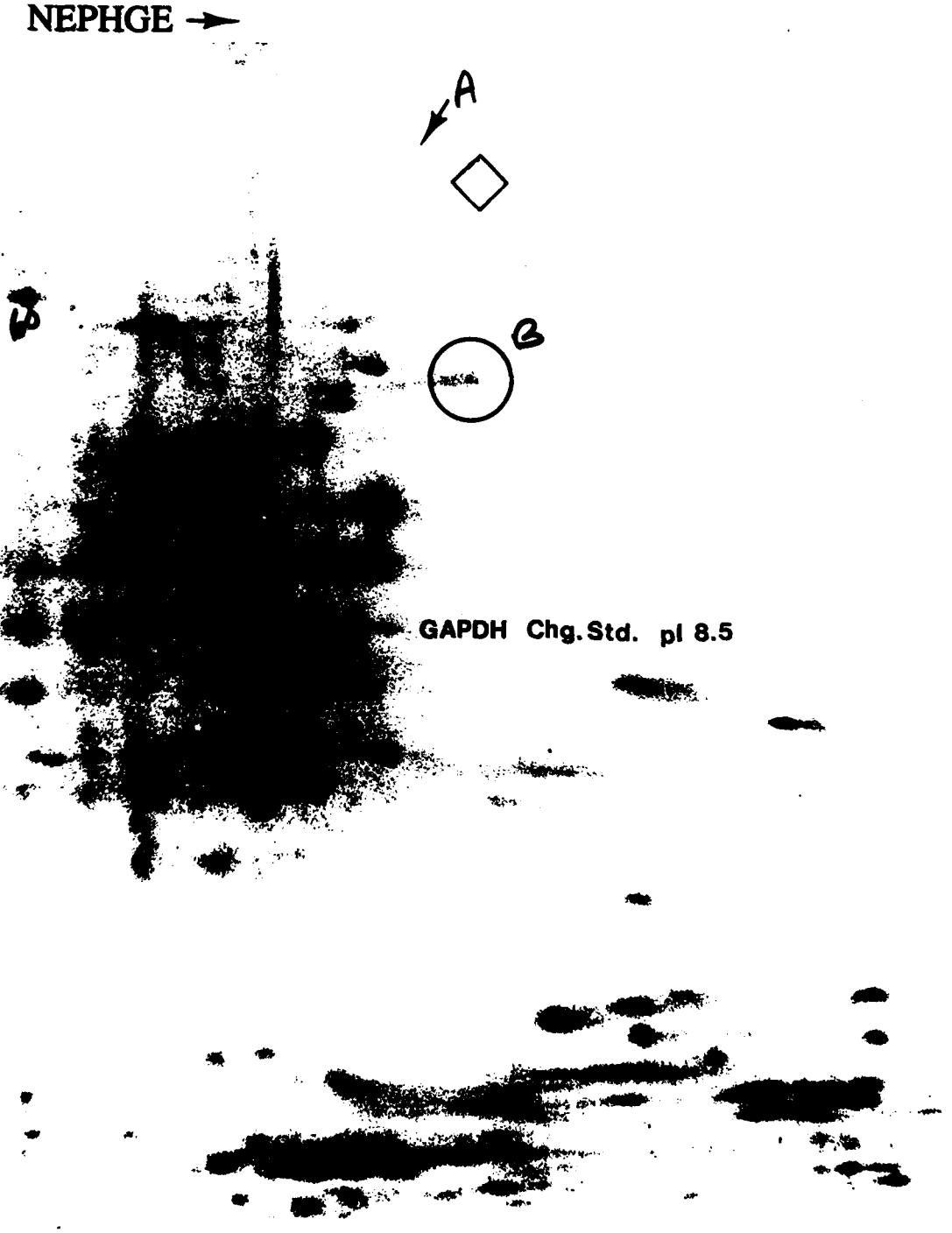
FIGURE 1.

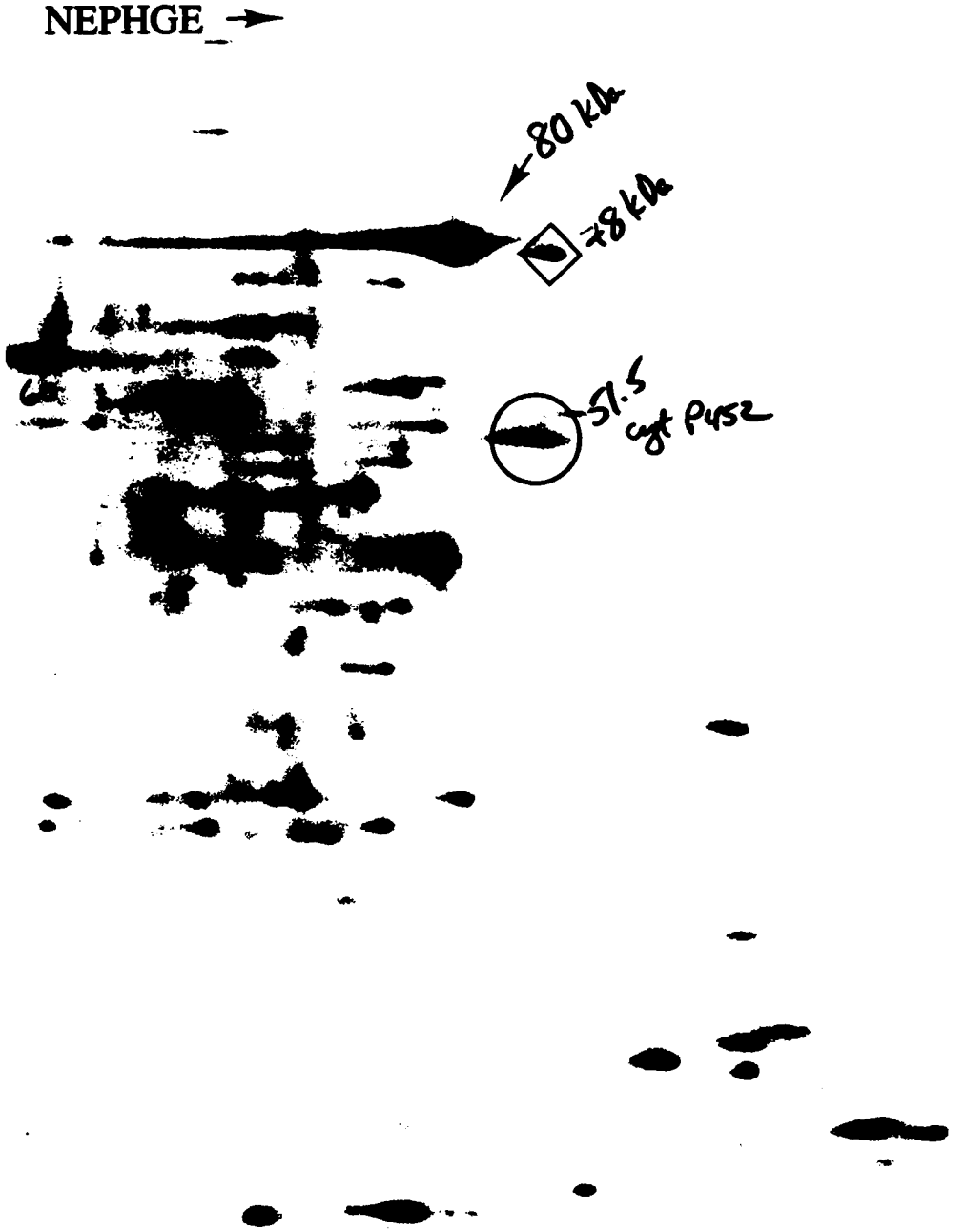
FIGURE 1.

FIGURE 2.



HEPATIC MICROSOMES - CONTROL

FIGURE 3.

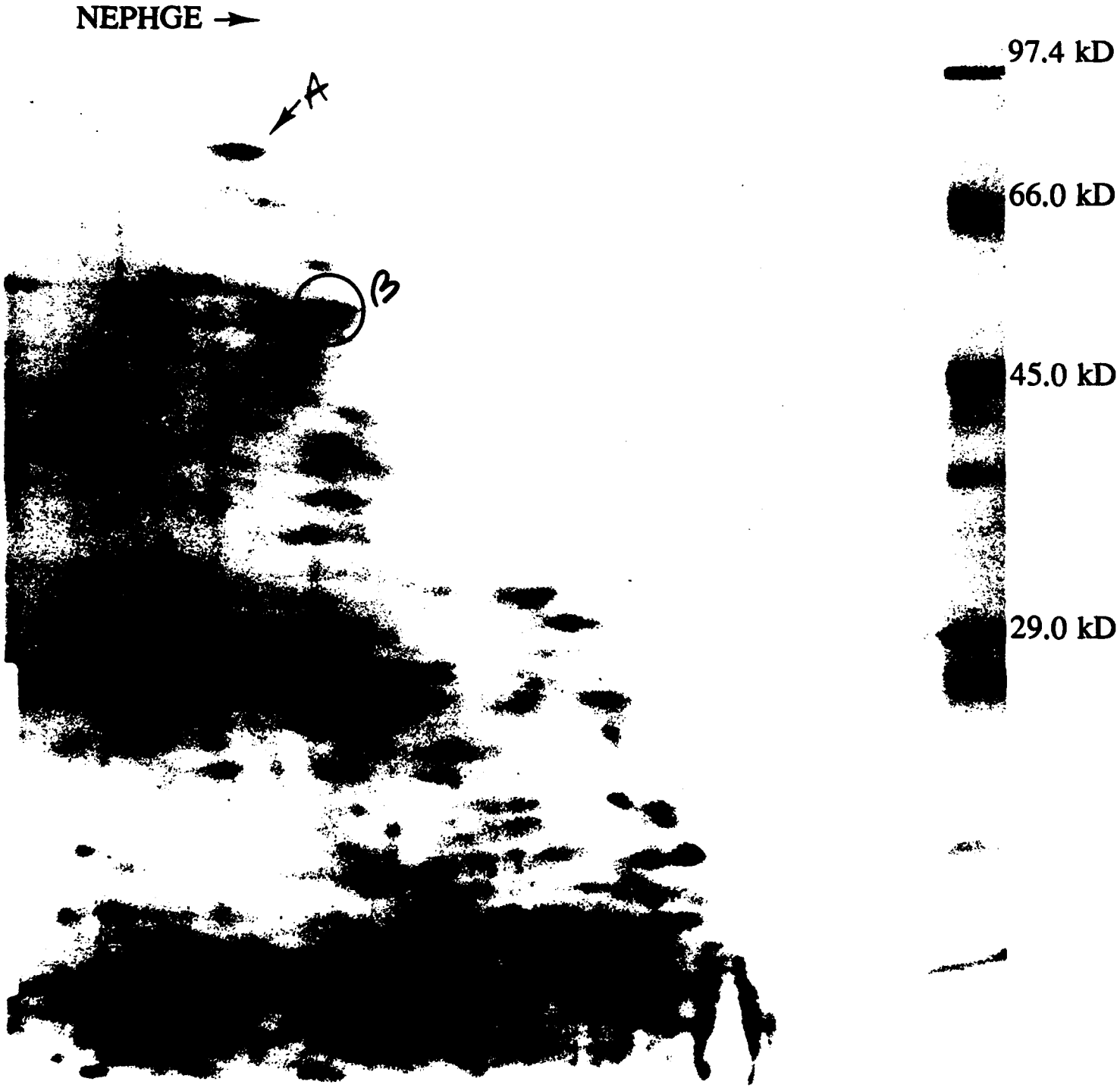


HEPATIC MICROSOMES - PFDA-TREATED 50mg/kg

FIGURE 4.

HEPATIC MICROSOMES - PFOA-TREATED 100mg/kg

FIGURE 5.

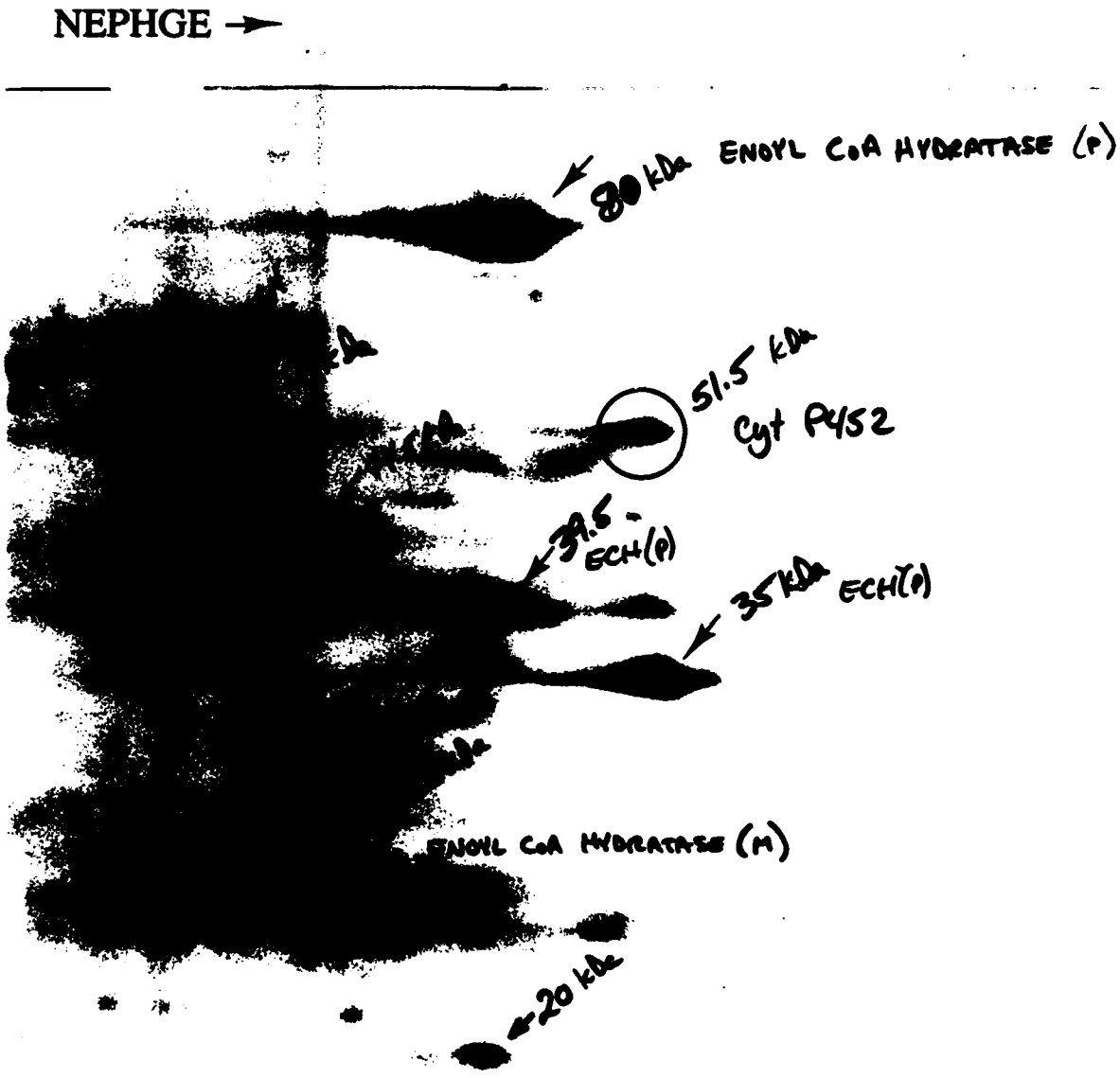


HEPATIC MICROSOMES - CLOFIBRATE-TREATED

FIGURE 6.

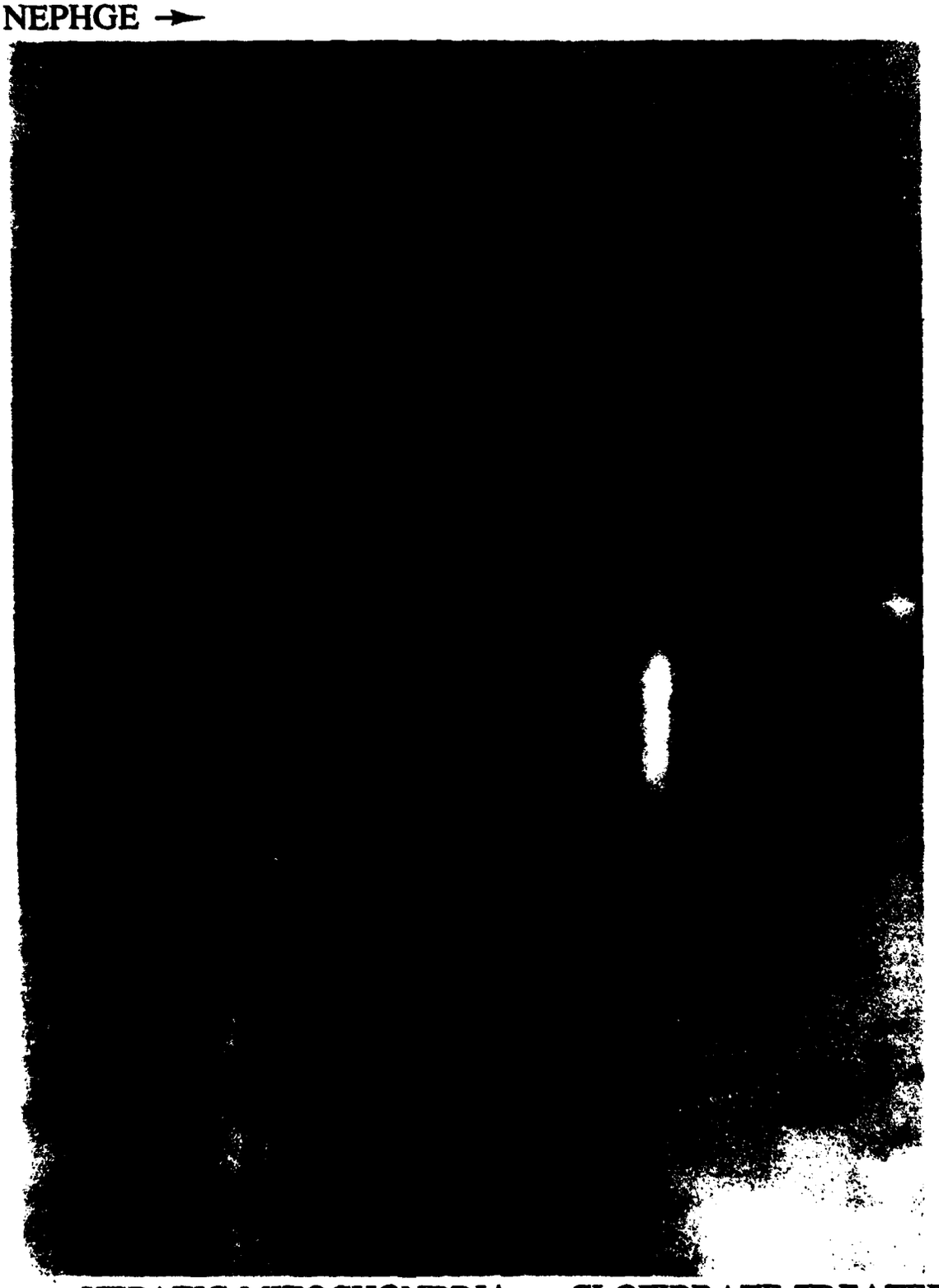
HEPATIC MITOCHONDRIA - CONTROL

FIGURE 7.



HEPATIC MITOCHONDRIA - PFDA-TREATED 50mg/kg

FIGURE 8.



HEPATIC MITOCHONDRIA - CLOFIBRATE-TREATED